AD_____

Award Number: W81XWH-06-1-0191

TITLE: Targeting Androgen Receptor Function by MicroRNA in Prostate Cancer

PRINCIPAL INVESTIGATOR: Girish C. Shukla, Ph.D.

CONTRACTING ORGANIZATION: Cleveland Clinic Foundation,
Cleveland, OH 44109

REPORT DATE: July 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-07-2007	Final	1 Jan 2006 – 30 Jun 2007

4. TITLE AND SUBTITLE

Targeting Androgen Receptor Function by MicroRNA in Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0191

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Girish C. Shukla, Ph.D.

Email: g.shukla@csuohio.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Cleveland Clinic Foundation,
Cleveland, OH 44195

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Prostate cancer is the most commonly diagnosed and second most deadly cancer in North American men and the blockade of androgen action through the AR has been the cornerstone of systemic therapy of prostate cancer. However, the effectiveness of this therapy is rather transient which inevitably fails and tumor growth resumes despite androgen blockade. The failure of AR receptor antagonists results in higher levels of AR protein which promotes the development of androgen-independent prostate cancer. Originally we proposed the utilization of micro (mi) RNAs to blockade the expression of AR in prostate carcinoma cells. We have identified a few miRNAs that can repress the AR protein synthesis in prostate carcinoma cells. Our long-term goals are to identify naturally occurring miRNAs that have potential to block the activity of AR and to improvise their efficacy by rational designing to provide novel 'AR Antagonist miRNAs'.

15. SUBJECT TERMS
Anti AR MiRNAs, noncoding RNA

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	6	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION:

Prostate cancer is the most commonly diagnosed and second most deadly cancer in North American men. Androgen receptor (AR) protein plays an important role in the development and progression of prostate cancer. Blockade of androgen action through the AR has been the cornerstone of systemic therapy of prostate cancer. However, the effectiveness of this therapy is rather transient which inevitably fails and tumor growth resumes despite androgen blockade. The failure of AR receptor antagonists results in higher levels of AR which is one of the causative factors of the development of androgen-independent prostate cancer. We proposed the utilization of micro (mi) RNAs to blockade the expression of AR in prostate carcinoma cells. Our goals are to identify naturally occurring miRNAs that affect the activity of AR and to improvise their efficacy by rational RNA-RNA interactions (between miRNA and 3' mRNA region of AR) to provide novel 'therapeutic miRNAs' which can target the expression of AR.

BODY:

Task 1. To examine if androgen receptor (AR) translation is modulated by a naturally occurring hsa-mir-183 microRNA (miRNA) and to validate that the 3'UTR of AR is a bona fide target of miRNA using chimeric luciferase reporter constructs (Months 1-12).

Task 1a Proposed Work: Design and commercial acquisition and subsequent overexpression of synthetic miRNAs by transient transfections into LNCaP cells and investigation of AR expression levels by Western blot using anti-AR antibodies. Confirmation that hsa-miR-183 is involved in AR translational control will be achieved (Months1-3).

Work Done: I transfected synthetic miRNAs to LNCaP cells and analyzed the translational repression of AR by Western Blotting. The activation of the AR expression was done with the addition of Dihydrotestosterone in cell culture. Immuno blots were performed on lysates prepared from miRNA transfected LNCaP cells. The transient transfection of synthetic hsa-miR-183 oligos did not repress AR protein levels which can be detected by immuno blots. Therefore, I decided to clone genomic regions spanning miRNA in an expression vector to overexpress in LNCaP cells.

In addition, I identified other miRNAs that have the potential to bind to 3' UTR of the AR thus to repress its expression. The results are shown in the bottom of this document.

Task 1b Proposed Work: Development of multiple test and control luciferase reporter expression constructs, each with an engineered 3' untranslated region (miRNA targeted sequences) of AR cloned downstream of the stop codon of luciferase cDNA. Chimeric luciferase and appropriate control reporter constructs will be ready (months 1-6).

Work Done: I have developed test and control luciferase reporter expression constructs containing 3' UTR of AR. I also have developed control reporter constructs but unable to test them in cell culture experiments yet.

Task 1c Proposed Work: Confirmation that AR 3' UTR sequences containing chimeric luciferase reporter constructs are responsive to ectopically expressed synthetic miRNAs. Optimization of reporter assays, evaluation of target specificities and interactions between target sequences and miRNAs will be achieved (Months 6-8).

Work Done: I have cloned Hsa-miR-183, Hsa-miR-124a and Hsa-miR-488 into lentiviral vectors for stable expression in LNCaP and CWR22RV1 cells. I have identified Hsa-miR-124a and Hsa-miR-488 by bioinformatics approaches after the start of this grant. We have reporter plasmids containing Hsa-miR-124a and

Hsa-miR-488 miRNA coding regions. However, these constructs require testing for AR repression in LNCaP cell culture experiments.

Task 1d Proposed Work: Rational design and testing of designer miRNAs that can modulate the expression of AR 3' UTR containing chimeric luciferase reporter constructs. (Months 8-10).

Work Done: This task was to be accomplished following to the identification of miRNAs that would inhibit AR expression maximally. However, due to my laboratory relocation and unavailability of funds I am unable to test constructs designed to express miRNAs from eukaryotic expression and Lentiviral vectors.

Task 2.
Proposed Work: Development of an inducible miRNA based cell culture model system to target translational knockdown of endogenous androgen receptor in prostate carcinoma LNCaP cells.

Work Done: This specific aim is primarily dependent on the Task 1d. In order to have inducible most effective miRNAs system, we needed to identify the miRNAs that were effective in blockading the AR expression in LNCaP cells. As stated above we have the reporter Lentiviral miRNA expressing plasmids to test their efficacy in AR inhibition.

PROBLEMS: Last year in August 2006 the PI relocated his laboratory from Cleveland Clinic Foundation to Cleveland State University. Since then, the PI is trying to have funds transferred to CSU which would allow him to continue working on the proposal and accomplish the required goals. Although, the PI was able to generate reporter plasmids required for to accomplish Aim 1 during the initial phase of this funding, but unable to test them. Due to the lack of funding and resources, I am unable to make any progress beyond the constructions of test miRNA expressing plasmids.

KEY RESEARCH ACCOMPLISHMENTS: A number of constructs containing miRNAs coding regions as well as AR target sequences are ready for testing. The target sequences expressing luciferase and miRNA expressing reporter constructs are available for testing the miRNA:AR target validation experiments. In addition, I also have cloned miRNAs into Lentiviral vectors that will be utilized to develop stable LNCaP cell lines expressing miR-183, miR-124 and miR-488. However, all these experiments are subjected to extension of the project and availability of funding.

REPORTABLE OUTCOMES: Unfortunately, we do not have reportable outcomes from this funding. However, to continue to build upon the knowledge and resources we have gained during this period I need extension and remaining funds. In addition, I have applied for New Investigator Award from PCRP.

CONCLUSION: We have developed reporter constructs expressing multiple miRNAs and have opportunity to test for their efficacy in AR inhibition in prostate cancer cells. The experimental identification of miRNAs which maximally repress the translational activity of the AR and further improvisation in their design will allow us to develop a subset of "AR antagonist miRNA".

SO WHAT: We hope to use these miRNAs to maximally and irreversibility blockades the expression of AR in prostate carcinoma cells in the initial phase. To demonstrate the effects and sensitivity of "AR Antagonist miRNA" subset we will express them in cell lines and xenograft these cells in to nude mouse model to evaluate their roles in proliferation and metastasis.

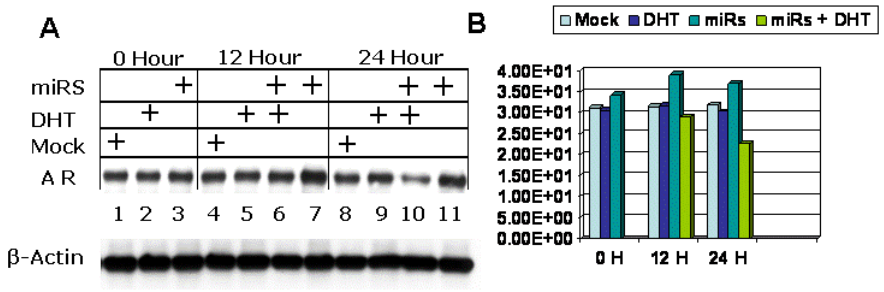

Figure 1: Downregualtion of Androgen Receptor by ectopic expression of miRNAs in Prostate Carcinoma cells LNCaP: Cell Treatment: *LNCaP prostate cancer cells were purchased from the ATCC. They were routinely cultured in RPMI medium supplemented with charcoal and dextran treated 10% fetal calf serum and antibiotics at 37^0C in an atmosphere of 5%CO_2. For treatment, $5x10^6$ cells were seeded in 10 cm cell culture plates. The 0 hour time point is actually the time point when cell were treated with DHT 24H after cells were grown in charcoal dextran treated serum containing medium. The cells were treated with DHT and miRNA were transfected at this time in RPMI 1640 medium.* ***Western Blot Analysis:*** *Cells were harvested by scraping and lysed in sample buffer. Equal amounts of protein were loaded onto 4–12% bis-tris-polyacrylamide gels, separated, and subsequently transferred onto nitrocellulose membranes. Blots were treated with 5% nonfat milk buffer for 1 H and incubated overnight at 4^0C with 1:1000-diluted mouse monoclonal anti–AR (Santa Cruz Biotechnology, CA). After multiple washings, membranes were incubated with a horseradish peroxidase–conjugated antimouse IgG (GE healthcare), for 1 H at room temperature. The blots were washed again and proteins were visualized with enhanced chemoluminescent substrate (GE healthcare). The bands were quantitated using densitomertic scans and ImageQuant program.*

Repression of AR translational activity by miRNA cocktail: We also have cloned genomic regions spanning miRNA genes in pcDNA 3.1 (-) vectors a and transfected into LNCaP cells using Calcium Phosphate method. Results are shown in Figure 1 As shown in Figure Panel A lane 10 and in the presence of DHT we found two fold reduction of total AR protein compared to 0H and 12H as quantitated by western blot and ImageQuant (Panel B). This experiment was repeated few times with consistent results. However, we observed the downregulation of AR protein only by the cotransfection of all miRNAs (cocktail of miR-183, miR-124a and miR-130) reporter plasmids. A single miRNA reporter construct failed to elicit any noticeable downregulation of AR in LNCaP cells (data not shown). We found two fold reduction of AR protein between 12 H to 24 H time points in the total lysates when all three miRNAs were cotransfected. This result demonstrates that the miRNAs that we identified in our initial screen can repress the expression of AR synergistically in combination; possibly by inhibiting the translational activity. However, they are unable to completely blockade the expression of AR at least at the protein level as evident by the western blot analysis. Further experiments are needed to confirm if the downregulation of AR is a direct effect of multiple miRNAs or “miRNA cocktail” and not an indirect consequence or bystander effect.